Hypoxic Conditioning Suppresses Nitric Oxide Production upon Myocardial Reperfusion

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Physiologically modulated concentrations of nitric oxide (NO) are generally beneficial, but excessive NO can injure myocardium by producing cytotoxic peroxynitrite. Recently we reported that intermittent, normobaric hypoxia conditioning (IHC) produced robust cardioprotection against infarction and lethal arrhythmias in a canine model of coronary occlusion-reperfusion. This study tested the hypothesis that IHC suppresses myocardial nitric oxide synthase (NOS) activity and thereby dampens explosive, excessive NO formation upon reperfusion of occluded coronary arteries. Mongrel dogs were conditioned by a 20 d program of IHC (FIO_2 9.5–10%; 5–10 min hypoxia/cycle, 5–8 cycles/d with intervening 4 min normoxia). One day later, ventricular myocardium was sampled for NOS activity assays, and immunoblot detection of the endothelial NOS isoform (eNOS). In separate experiments, myocardial nitrite (NO_2^-) release, an index of NO formation, was measured at baseline and during reperfusion following 1 h occlusion of the left anterior descending coronary artery (LAD). Values in IHC dogs were compared with respective values in non-conditioned, control dogs. IHC lowered left and right ventricular NOS activities by 60%, from 100–115 to 40–45 mU/g protein ($P < 0.01$), and decreased eNOS content by 30% ($P < 0.05$). IHC dampened cumulative NO_2^- release during the first 5 min reperfusion from 32 ± 7 to 14 ± 2 μmol/g ($P < 0.05$), but did not alter hyperemic LAD flow (15 ± 2 vs. 13 ± 2 ml/g). Thus, IHC suppressed myocardial NOS activity, eNOS content, and excessive NO formation upon reperfusion without compromising reactive hyperemia. Attenuation of the NOS/NO system may contribute to IHC-induced protection of myocardium from ischemia-reperfusion injury. Exp Biol Med 233:766–774, 2008

Key words: nitric oxide synthase; cardioprotection; intermittent hypoxia; dogs; myocardial ischemia

Introduction

Restoration of coronary blood flow is the most effective means of salvaging ischemic myocardium, but reperfusion can paradoxically exacerbate ischemic injury (1). Ischemia alters the chemical composition of cells, creating an environment in which the reintroduction of oxygen upon reperfusion precipitates inflammation and oxidative stress, compromising recovery of cellular function and even causing cell death. With its high metabolic demand, myocardium ranks among the tissues most susceptible to ischemia/reperfusion (I/R) injury. Myocardial I/R injury can occur in a variety of clinical settings including balloon angioplasty, thrombolytic therapy, coronary artery bypass grafting, and heart transplantation.

Recent studies in our laboratory (2, 3) demonstrated the cardioprotective potential of intermittent, normobaric hypoxia conditioning (IHC). Dogs were conditioned by a 20-day program of brief (5–10 min) hypoxia exposures interspersed with periods of reoxygenation. IHC produced dramatic reductions in myocardial infarct size and ventricular arrhythmias during coronary artery occlusion-reperfusion experiments conducted one day after completing the IHC program (2). This potent cardioprotection developed progressively over the course of the 20 d IHC program, since a single IHC session imparted no cardioprotection against infarction or arrhythmias, and the protection afforded by a 10 d IHC program, although appreciable, was less complete than that provided by the full 20 d program (3). The progressive development of the cardioprotection suggested that changes in gene expression and protein content may have produced the cardioprotected phenotype.

Myocardial nitric oxide (NO) production increases

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during ischemia (4–7). Moderate NO production can be beneficial: it relaxes smooth muscle to increase tissue perfusion, suppresses platelet aggregation and leukocyte adherence to endothelium, and dampens fibrinogen formation. However, excessive NO formation can injure ischemic myocardium by activating pro-apoptotic transcription factors (8), inhibiting metabolic enzymes (9) and/or producing cytotoxic peroxynitrite upon reperfusion (10). Administration of NO or NO donors prior to acute myocardial ischemia has been demonstrated to evoke cardioprotection against I/R injury (11), but excessive NO concentrations induce necrosis and apoptosis in myocardium (12–14). Hearts isolated from transgenic endothelial NOS (eNOS) knockout mice and subjected to global ischemia-reperfusion had improved post-ischemic recovery of contractile performance and myocardial phosphocreatine concentration vs. hearts of wild-type mice (15). Moreover, 30 min coronary occlusion and 48 h reperfusion produced appreciably smaller myocardial infarcts in eNOS knockout than wild-type mice (16).

This study was conducted in mongrel dogs to test the hypothesis that IHC evokes potentially cardioprotective adaptations of the myocardial NOS system. Specifically, the proposal that IHC suppresses myocardial NOS activity sufficiently to dampen the toxic burst of excessive NO formation upon reperfusion of ischemic myocardium was tested.

Methods

Animals. Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center and was conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (National Institutes of Health, publication no. 85–23, revised 1996). Healthy adult mongrel dogs were assigned to IHC and non-conditioned control groups. The IHC dogs completed a 20-day IHC program (2) by breathing hypoxic atmospheres in a 270 liter acrylic chamber. Daily sessions consisted of 5–8 cycles of hypoxia (FIO_2 9.5–10%), each 5–10 min, with intervening 4 min exposures to room air. Dogs in the control group included 4 sham-conditioned dogs that underwent 20 d of exposure to 21% O_2 within the hypoxia chamber, and 5 dogs subjected to neither IHC nor sham conditioning. In our recent studies in this model (3), these two groups had nearly identical severity of ischemic injury following the coronary artery occlusion-reperfusion protocol, as judged from infarct size/area at risk (nonconditioned dogs 39 ± 9%; sham-conditioned dogs 38 ± 6%) and arrhythmia scores (nonconditioned dogs 3.5 ± 0.5; sham-conditioned dogs 3.6 ± 0.4). Moreover, left ventricular NOS activity was very similar in the two groups (nonconditioned dogs 100 ± 14 nmol/min/g protein; sham-conditioned dogs 109 ± 16 nmol/min/g protein). Based on the similar values of these key variables, we elected to combine these two non-hypoxic subgroups. Dogs were maintained on a 12:12-hr light:dark cycle and received standard chow diet and water *ad libitum* throughout the IHC and sham conditioning programs. The dogs were fasted overnight before the terminal ischemia-reperfusion experiment.

Surgical Preparation and Myocardial Ischemia-Reperfusion Protocol. Coronary artery occlusion-reperfusion experiments were conducted in IHC dogs ($n = 6$) one day after completing the IHC program, and in non-IHC, control dogs ($n = 5$). After an overnight fast, dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv), intubated, and mechanically ventilated with room air. A femoral vein was cannulated to administer $NaHCO_3$ and supplemental pentobarbital. A saline filled catheter was introduced into the thoracic aorta via a femoral artery to measure aortic pressure and to sample arterial blood for measuring hematocrit, hemoglobin, blood gases (2, 3) and nitrite (NO_2^-). Arterial pO_2, pCO_2, and pH were maintained within normal limits by ventilating with supplemental O_2, adjusting tidal volume and respiratory rate, and by administering $NaHCO_3$.

The heart was exposed through a left thoracotomy in the fifth intercostal space. The left anterior descending coronary artery (LAD) was isolated distal to its first major diagonal branch. An electromagnetic flow probe (Transonic Systems model 2SB, Ithaca, NY) was placed on the LAD to monitor coronary flow, and a silk suture was passed around the LAD immediately distal to the flow probe, forming a snare. After heparin administration (500 U/kg) the interventricular coronary vein, which selectively drains the LAD perfusion territory (17), was cannulated to sample coronary venous blood. Body temperature was measured with an intramuscular needle probe placed in the *quadriceps femoris* and maintained at 36.5–37.5°C by a circulating H_2O heating pad positioned under the dog.

The LAD occlusion-reperfusion protocol commenced after completion of surgery and stabilization of hemodynamic and blood gas variables. The LAD was occluded for 1 h by tightening the snare, and then reperfused for 5 h by releasing the snare (2). Ventricular fibrillation occurred in two control dogs following reperfusion; direct current countershocks (10 J) were applied to the epicardium within 15 s, by use of internal paddles, to restore sinus rhythm (3). Systemic arterial and coronary venous blood samples for measuring NO_2^- were collected just before LAD occlusion and during the first 40 min of reperfusion.

Nitrite Measurement. Myocardial release of NO_2^-, a stable product of NO oxidation (18), was measured as an index of NO formation (19, 20). NO_2^- concentrations in aortic and coronary venous plasma were measured by the Griess reaction, using a commercially available kit (Endogen, Rockford, IL). Plasma samples obtained after centrifugal sedimentation of formed elements were mixed with 1 vol reagent diluent and filtered (10 kD molecular weight cut off) by centrifugation at 14,000 *g* for 30 min at room temperature. Fifty μl aliquots of filtrate were pipetted

onto a 96-well plate. Griess reagents I and II were added, the plate was incubated for 10 min at room temperature, and then absorbance at 540 nm was measured in a Power Wave XS plate reader (Bio Tek, Winooski, VT). NO_2^- release was calculated by multiplying LAD flow times the arteriovenous difference in NO_2^- concentration, and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$.

Extraction and Measurement of Myocardial Enzymes. Enzymes were measured in myocardial biopsies taken from 8 IHC dogs one day after completing the IHC program, and from 4 control dogs. The dogs were anesthetized as described above, and the heart exposed through a left thoractomy. LAD occlusion-reperfusion experiments were not conducted in these dogs. Transmural biopsies of LAD- and left circumflex (LCX)-perfused left ventricle and of right ventricle were excised, quickly rinsed in cold saline, blotted and flash-frozen in liquid nitrogen. The frozen tissues were pulverized under liquid nitrogen using a mortar and pestle. Powdered tissue was extracted by homogenization in 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM ADP, 10 mM glutathione and 10 mM ethylenediaminetetraacetic acid (EDTA). Extract was centrifuged at $100{,}000 \cdot g$ for 20 min. The pellet was resuspended in phosphate buffer and re-extracted twice. The three supernatant fractions were combined, divided into aliquots, and stored at –80°C.

Total NOS activity in tissue extracts was determined from the Griess reaction, with a commercially available kit (Oxis International, Portland, OR). Tissue extract was combined with assay buffer on 96-well plates. A 1 mM NADPH solution and nitrate reductase were added sequentially according to kit instructions. After 60 min of incubation, 10 μl aliquots of cofactor and lactate dehydrogenase solutions were added to each well. Twenty minutes later, Griess Reagents I and II were added. After another 10 min incubation, the plate was read at 540 nm. It should be noted that NOS inhibitors were not applied to test for non-enzymatic nitrite formation in this assay. Activities of lactate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphofructokinase, and creatine kinase were measured by standard colorimetric assays (21). Extract protein concentration was measured according to Bradford (22) for normalization of enzyme activities.

Immunoblot Assessment of eNOS Content and Phosphorylation. Endothelial NOS was analyzed by immunoblotting of myocardial extracts. Samples containing 20 μg protein were resolved by electrophoresis (100 V for 2 h) on 10% polyacrylamide gels. Proteins were electrophoretically transferred (350 V for 3 h) from the gels to nitrocellulose membranes. Membranes were incubated in 5% nonfat milk for 2 h to block non-specific binding sites. Source gels were stained with Coomassie blue to confirm electrophoretic transfer.

Rabbit anti-eNOS (Stressgen, Victoria, BC) and anti-iNOS (BD Bioscience) polyclonal antibodies were used to detect eNOS and iNOS. Mouse anti-$P^{1177}S$ eNOS and anti-$P^{495}T$ eNOS antibodies (BD Bioscience) were used to detect eNOS phosphorylation. Immune complexes were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) upon exposure to X-ray film. Human endothelial cell lysates provided a positive control for eNOS. The membranes were stripped and re-probed with rabbit anti-actin antibody (Stressgen) to detect actin, which provided a loading control. Protein band densities were quantified with analytical software (AlphaEaseFC 4.0, Alpha Innotech, San Leandro, CA). Band densities of total eNOS, $P^{1177}S$ eNOS, $P^{495}T$ eNOS, and iNOS were normalized to actin band density, and band densities of phosphorylated eNOS were normalized to total eNOS band densities.

Table 1. Arterial Hematocrit and Hemoglobin Content[a]

Group	*n*	Hematocrit (%)	Hemoglobin (g/dl)
IHC	14	38 ± 1	12.7 ± 0.3
Non-conditioned	9	40 ± 1	13.5 ± 0.4

[a] Values in intermittent hypoxia conditioned (IHC) dogs were measured on the day after completion of the 20-day IHC program. Values are means ± SEM. No statistically significant differences were detected.

Statistical Analyses. Data are expressed as mean ± standard error. Single comparisons of means between control and IHC groups were performed by applying two-tailed, unpaired Student's *t* tests. Repeated measurements of NO_2^- release and LAD flow were compared by one-way analyses of variance (ANOVA). When ANOVA detected statistically significant differences, Tukey's test was applied to identify specific differences between mean values. *P* values < 0.05 were taken to indicate statistical significance.

Results

Hematocrit and Hemoglobin Content of Arterial Blood. Hematocrit and hemoglobin content were measured in arterial blood sampled one day after completing the IHC program ($n = 12$), and in non-IHC control dogs ($n = 10$). The IHC program did not alter hematocrit or hemoglobin content (Table 1).

Activities of Myocardial Enzymes. Four enzymes known to be induced by sustained hypoxia, lactate dehydrogenase (LDH), glucose 6-phosphate dehydrogenase (G6PDH), creatine kinase (CK), and phosphofructokinase (PFK) (23–26), were measured in LAD- and left circumflex coronary artery (LCX) perfused regions of left ventricular myocardium, and in right ventricular myocardium of 8 IHC dogs and of 4 control dogs (Fig. 1). No statistically significant treatment effects on these enzyme activities were detected in any perfusion territory, although a trend toward increased G6PDH activity in the LAD-perfused region of IHC vs. control dogs was noted.

NO Production During Myocardial Reperfu-

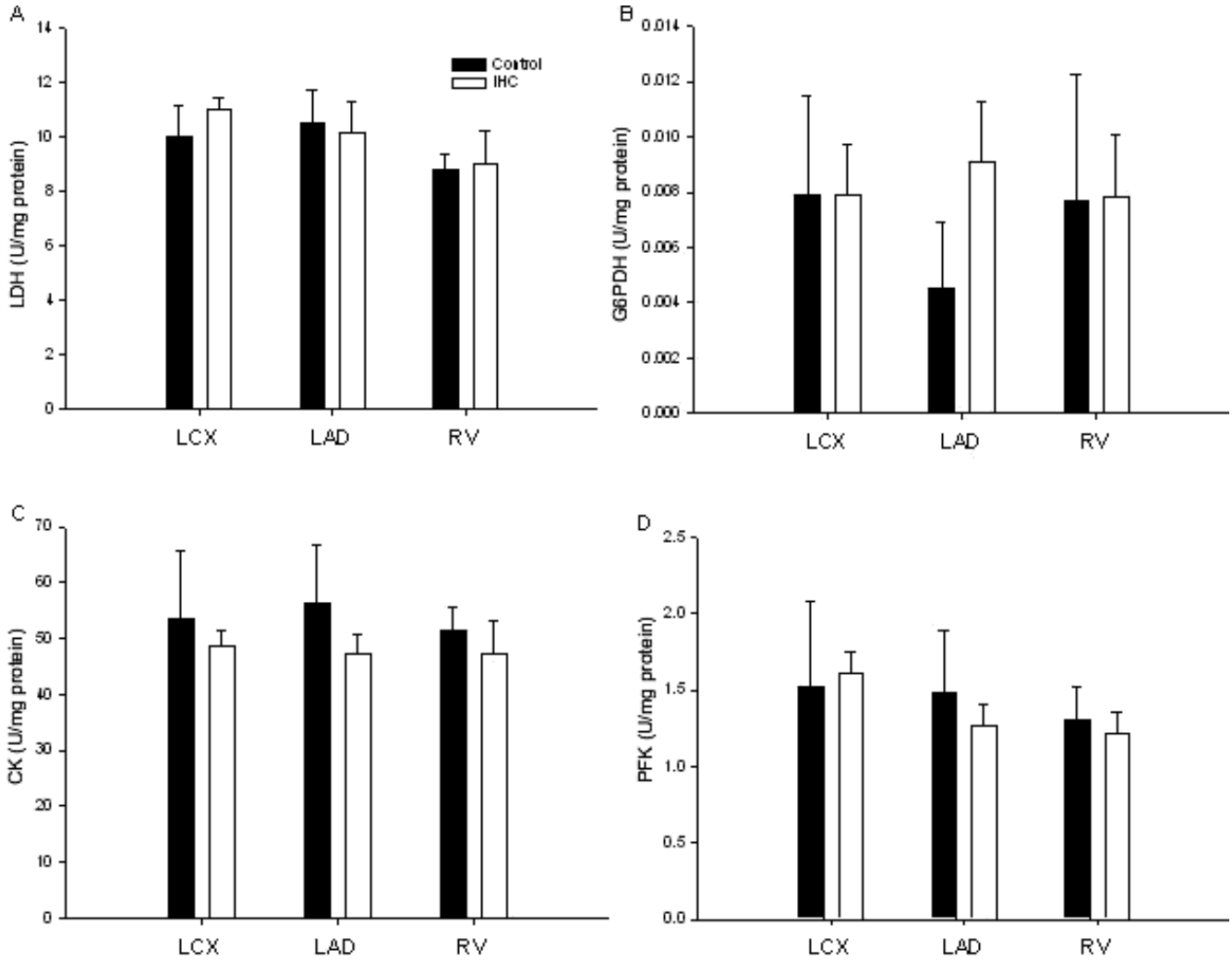

Figure 1. IHC did not alter activities of hypoxia-inducible myocardial enzymes. Lactate dehydrogenase (LDH), glucose-6-phophate dehydrogenase (G6PDH), creatine kinase (CK), and phosphofructokinase (PFK) activities were measured in transmural biopsies taken from left ventricular myocardium perfused by the left anterior descending (LAD) and left circumflex (LCX) coronary arteries, and from right ventricular myocardium (RV) of non-hypoxic controls (filled bars) and dogs conditioned by 20 d IHC (open bars). Values are means ± SEM from 4 control and 8 IHC dogs. No statistically significant between-group differences were detected.

sion. Nitrite (NO_2^-) was measured to assess NO formation in the LAD-perfused myocardium before and after LAD occlusion (Fig. 2A). Pre-ischemic NO_2^- release did not differ among the IHC ($n = 5$) vs. control groups ($n = 5$; $P = 0.41$). Following release of the LAD occlusion, NO_2^- release increased sharply and peaked at 2 min reperfusion in the control group, and then subsided (Fig. 2A). In contrast, there was not a statistically significant surge in NO_2^- release upon reperfusion in the IHC group. During the initial 5 min of reperfusion, the cumulative myocardial NO_2^- release of the IHC group was less than half that of the control group (14 ± 2 vs. 32 ± 7 $\mu mol \cdot g^{-1}$; $P = 0.017$; Fig. 2B).

A robust hyperemia occurred following LAD reperfusion, which gradually subsided between 5 and 15 min reperfusion (Fig. 3A). IHC did not attenuate the initial reperfusion hyperemia; indeed, cumulative LAD flow during the first 5 min reperfusion in the IHC group was comparable to that of the control group (Fig. 3B). IHC did tend to lower LAD flow at 5–10 min reperfusion (Fig. 3A), although this effect was not statistically significant and occurred after the period in which IHC dampened NO_2^- release (Fig. 2A).

Nitric Oxide Synthase Activity. NOS activity was measured in the same myocardial biopsies in which other enzyme activities were measured. IHC lowered NOS activity in the LAD and LCX perfusion territories of the left ventricle, and in right ventricular myocardium by 55–63% (Fig. 4). These IHC-induced reductions in NOS activity paralleled the 56% reduction in NO_2^- formation during reperfusion hyperemia (Fig. 2B).

Endothelial NOS Content and Phosphorylation. Immunoblotting revealed an IHC-induced 30% decrease in left ventricular myocardial eNOS content, the principal constitutive NOS isoform in myocardium

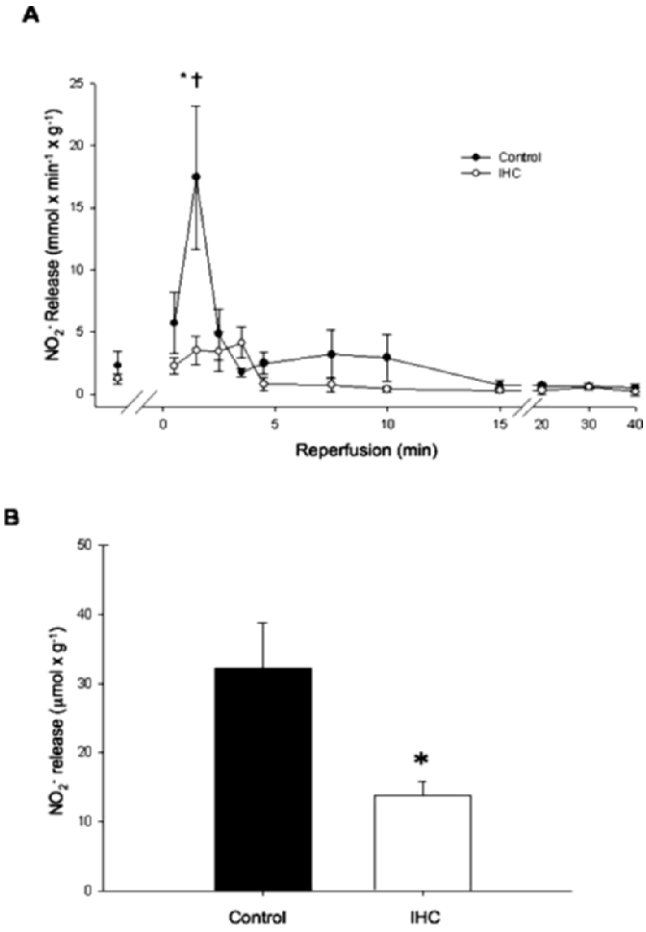

Figure 2. Nitrite release during reperfusion reactive hyperemia. NO_2^- release peaked within 2 min reperfusion in non-hypoxic control dogs (Panel A). IHC dampened this reperfusion NO_2^- burst, and lowered cumulative NO_2^- release during the first 5 min reperfusion by 56% (Panel B). Means ± SEM from 6 IHC and 5 control experiments. * $P < 0.05$ vs. non-hypoxic control, † $P < 0.05$ vs. pre-occlusion baseline (BL).

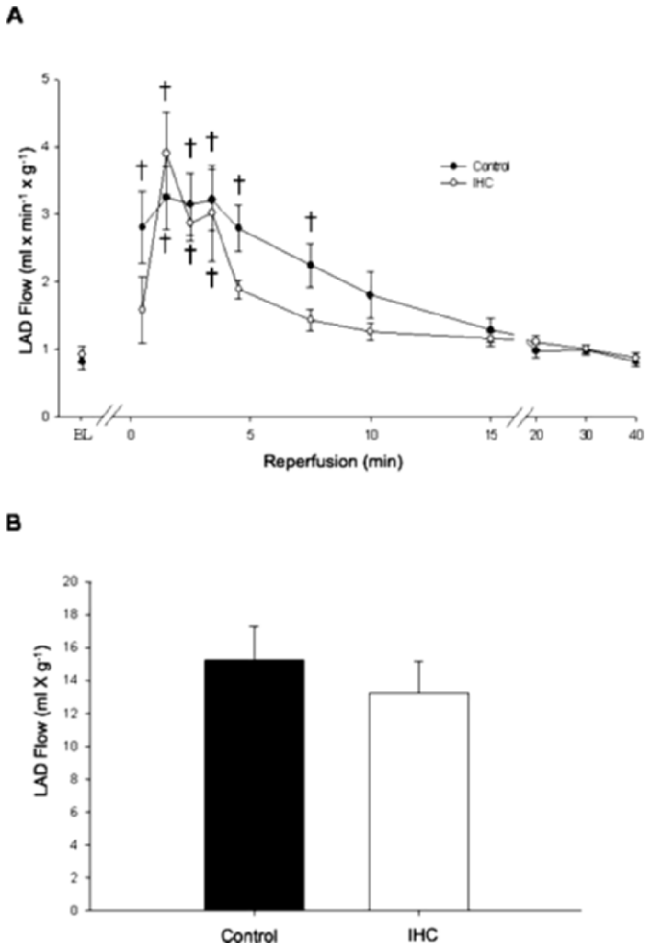

Figure 3. LAD blood flow during reperfusion. No significant differences were detected in LAD flow during pre-occlusion baseline or following reperfusion (Panel A). Cumulative LAD flow during the first 5 min reperfusion did not differ in non-hypoxic vs. IHC dogs (Panel B). Means ± SEM from the same experiments as in Figure 2. † $P < 0.05$ vs. baseline (BL).

(Fig. 5A). eNOS activity is modulated by phosphorylation at ^{1177}S, which increases eNOS activity, or at ^{495}T, which inactivates the enzyme (27). When normalized to total eNOS content, ^{1177}S phosphorylation was sharply increased in the IHC vs. control group, but ^{495}T phosphorylation was unaltered by IHC (Fig. 5B).

Discussion

Recently we demonstrated remarkable cardioprotection evoked by a 20 d IHC program (2, 3). One-hour occlusion of the LAD followed by 5 h reperfusion infarcted nearly 40% of the ischemic myocardium of control dogs, but only 1% in the IHC dogs. Moreover, IHC prevented ventricular tachycardia and fibrillation that occurred upon LAD reperfusion in 82% of the non-hypoxic dogs (3). The gradual development of this potent cardioprotection over the course of the 20 d IHC program (3) suggested that progressive changes in myocardial contents of potentially

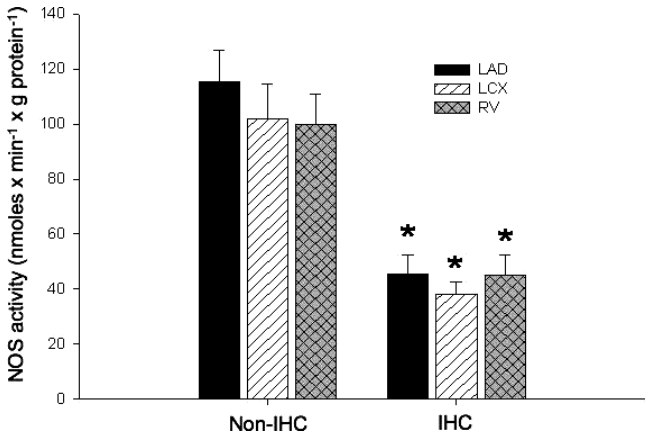

Figure 4. IHC suppressed nitric oxide synthase activities in left and right ventricular myocardium. NOS activities were measured in the same myocardial biopsies as in Figure 1. Abbreviations as in Figure 1. * $P < 0.05$ vs. non-hypoxic control.

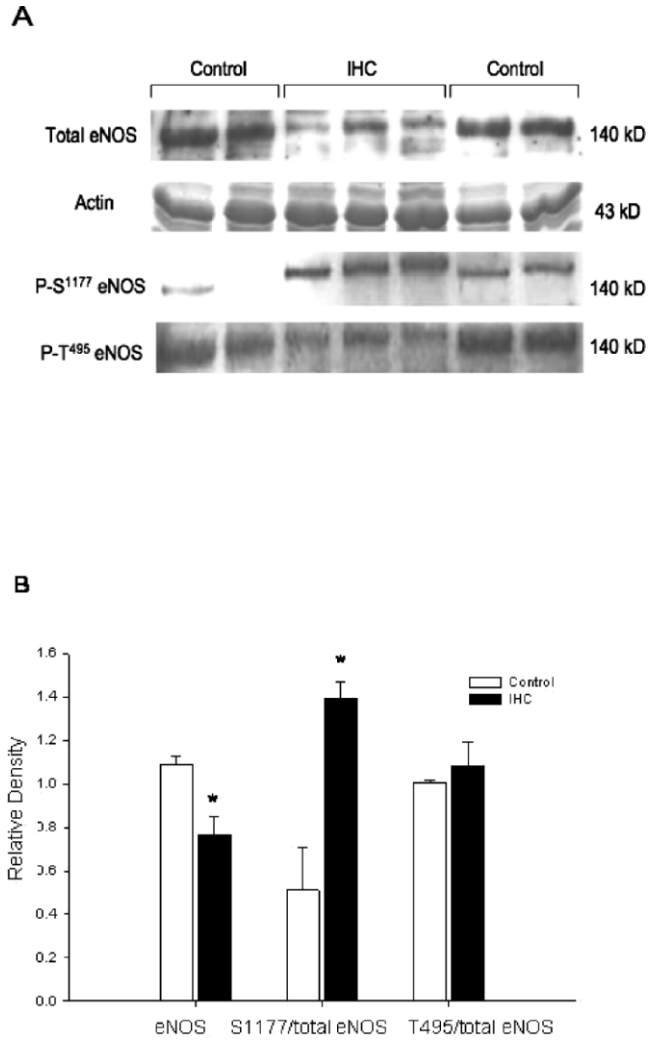

Figure 5. Immunoblot of eNOS in left ventricular myocardium of non-hypoxic and IHC dogs. Densities of the bands for total eNOS, P-^{1177}S eNOS, and P-^{495}T eNOS are normalized to densities of the actin bands, which served as loading controls. Values in panel B are means ± SEM from 8 IHC and 4 non-hypoxic control experiments. * $P < 0.05$ vs. control.

protective or adverse proteins may have contributed to the protection.

Since IHC might stimulate hypoxia inducible factor-1 (HIF-1)-dependent transcription, we examined a panel of potentially cardioprotective enzymes expressed in response to HIF-1 (24, 25). In contrast to chronic hypoxia, which decreases activities of CK (26) and increases G6PDH, LDH and PFK activities in rat myocardium (23, 24), IHC did not alter these enzyme activities in canine myocardium. Chronic hypoxia in rats substantially increases hematocrit (29), due to erythrocyte production stimulated by erythropoietin, a HIF-1 responsive gene product (30). On the other hand, the present IHC regimen in dogs increased neither hematocrit nor blood hemoglobin content. These divergent adaptive responses to chronic and intermittent hypoxic conditioning could conceivably be related to differences in hypoxic 'dose,' i.e. intensity · duration of hypoxia, which correlates with the extent of HIF-1 activation (10, 31). Thus, any induction of HIF-1 by IHC was not sufficiently intense or prolonged to evoke HIF-1-mediated erythropoiesis or enzyme synthesis.

Favorable vs. Detrimental Effects of Nitric Oxide. A physiological vasodilator and trigger of ischemic and pharmacological preconditioning (32), NO is another putative mediator of IHC induced cardioprotection. Administration of NO or NO donors prior to severe ischemia has been found to reduce I/R injury, including cardiomyocyte apoptosis (33, 34). NO increases cardiomyocyte function by inhibiting phosphodiesterase III, thereby increasing cyclic AMP concentration and activating protein kinase A (35). Moreover, physiological concentrations of NO might increase myocardial function by directly activating voltage gated sarcolemmal Ca^{2+} channels or Ca^{2+} dependent Ca^{2+} release channels in sarcoplasmic reticulum (36). Other potential cardioprotective actions of NO include coronary vasodilation (37), suppression of inflammatory neutrophil infiltration (38), preservation of coronary endothelial function (39), and reduction of myocardial O_2 demand (40–42). NO can decrease mitochondrial Ca^{2+} uptake by activating mitochondrial ATP dependent K^+ channels (43, 44), thereby ameliorating mitochondrial Ca^{2+} overload.

High concentrations of NO can contribute to myocardial ischemia-reperfusion injury, primarily by generating highly reactive NO derivatives (10). NO rapidly and irreversibly condenses with the superoxide radical $\cdot O_2^-$ (45) to generate peroxynitrite ($ONOO^-$), the precursor of a host of cytotoxic ROS and reactive nitrogen species (10). NO and $ONOO^-$ may directly attack catalytic Fe·S-centers of respiratory chain components and the Krebs cycle enzyme aconitase, and thereby impair ATP production (18, 46–48). High NO concentrations also initiate apoptotic cell death (12, 13) by activating mitogen-activated protein kinases and pro-apoptotic transcription factors (13). Reactive nitrogen species including NO and $ONOO^-$ are thought to contribute to cardiac sympathovagal imbalances in the brainstem and activation of cardiomyocytes, which may predispose to arrhythmia (49). $ONOO^-$ initiates peroxidation of membrane phospholipids (50).

Ischemia-reperfusion creates conditions that favor formation in myocardium of NO and its toxic derivative $ONOO^-$. The intense reactive hyperemia upon myocardial reperfusion produces shear stress, a major stimulus of NO formation by eNOS (51). Moreover, changes in the intracellular milieu during ischemia lead to explosive formation of $\cdot O_2^-$ when O_2^- is reintroduced upon reperfusion of myocardium (52–54). Oxidative stress imposed by ischemia-reperfusion can uncouple eNOS, causing the enzyme to release $\cdot O_2^-$ instead of NO by disrupting the normal flow of electrons from NADPH to *L*-arginine (55). On the other hand, decreased eNOS content would lower the potential for $\cdot O_2^-$ formation by the uncoupled enzyme. Thus, myocardial ischemia-reperfusion enhances formation of both NO and $\cdot O_2^-$, yet IHC-induced reductions in eNOS

content could dampen formation of NO from normally functioning eNOS and $\cdot O_2^-$ from the uncoupled enzyme, and thereby lessen $ONOO^-$ formation following reperfusion.

Mechanism of Intermittent Hypoxia Suppression of Myocardial NOS. The 20-day IHC program lowered myocardial NOS activity and eNOS content, and produced a commensurate dampening of nitrite release during the first 5 min of reperfusion, indicating decreased NO formation. Notably, the reduction in NO formation did not attenuate reactive hyperemia; thus, it appears that high concentrations of NO are not mandatory for full coronary vasodilation upon reperfusion.

Chronic and intermittent hypoxic exposures produce directionally opposite changes in NOS activity. Thus, chronic hypoxia increased eNOS activity of rat myocardium (56, 57), but intermittent hypoxia dampened rat myocardial eNOS activity (58). Chronic hypoxia increases expression of erythropoietin via HIF-1 activation (32). By increasing hematocrit, erythropoietin enhances intravascular shear stress on endothelium, thereby activating eNOS. However, when chronic hypoxia did not increase hematocrit, erythropoietin lowered eNOS activity (59). The 20 d IHC program tested here does not increase hematocrit (Table 1) (2, 3).

A second possible mechanism of IHC suppression of NOS may involve hypoxic enhancement of the nitrite reductase activity of deoxyhemoglobin. In the IHC paradigm, arterial hemoglobin O_2 saturation falls to roughly 70–75% during each hypoxia cycle (3). Deoxyhemoglobin catalyzes reduction of nitrite to NO, which escapes the erythrocytes in sufficient quantities to activate the endothelium-dependent vasodilatory mechanism during hypoxia (60–62). Conceivably, deoxyhemoglobin may have generated sufficient NO during the cyclic bouts of hypoxia to suppress NOS expression (62), causing myocardial eNOS content to progressively decline over the course of the IHC program.

Endothelial NOS activity is modulated by phosphorylation. Although total myocardial eNOS content fell, IHC increased fractional eNOS phosphorylation at ^{1177}S, a post-translational modification that enhances eNOS activity (27). There was no difference in ^{495}T phosphorylation, a repressor of the enzyme's activity (60). By activating eNOS, the increased ^{1177}S phosphorylation may have at least partially compensated for the IHC-induced reduction in eNOS content. In that case, it is possible that IHC may have adaptively suppressed ischemic induction of the inducible NOS isoform, which could conceivably decrease NO formation during reperfusion hyperemia.

In a recent, related study, Zhao *et al*. (16) found that expression of iNOS mRNA and protein was reduced in postischemic myocardium of eNOS knockout mice. Nitrotryosine staining indicated that peroxynitrite was also reduced, consistent with an observed reduction in infarct size. Thus, it is possible that the cardioprotective effects observed earlier in eNOS knockout mice by Flőgel *et al*. (15) were mediated by reduced iNOS. In any case, these findings support the conclusion of the current study that IHC is cardioprotective by dampening postischemic excessive NO production.

Summary. A 20 d IHC program recently shown to afford remarkable cardioprotection from ischemia-reperfusion injury (2, 3) suppressed canine myocardial NOS activity, eNOS content, and reperfusion NO release, without compromising reactive hyperemia. Other, potentially cardioprotective enzymes were not altered by IHC. Collectively, these results support the proposal that IHC-induced dampening of NO production upon reperfusion could contribute to cardioprotection afforded by IHC against the ravages of ischemia-reperfusion.

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